



**University of
Zurich**^{UZH}

**Zurich Open Repository and
Archive**

University of Zurich
University Library
Strickhofstrasse 39
CH-8057 Zurich
www.zora.uzh.ch

Year: 2019

Virus-like particles in a new vaccination approach against infectious laryngotracheitis

Schädler, Julia ; Sigrist, Brigitte ; Meier, Simone Martina ; Albini, Sarah ; Wolfrum, Nina

Abstract: Gallid alphaherpesvirus 1 (syn. infectious laryngotracheitis virus; ILTV) is the causative agent of infectious laryngotracheitis, a respiratory disease of chickens causing substantial economic losses in the poultry industry every year. Currently, the most efficient way to achieve protection against infection is immunization with live-attenuated vaccines. However, this vaccination strategy entails the risk of generating new pathogenic viruses resulting from spontaneous mutations or from recombination with field strains. This work presents a new approach based on virus-like particles (VLPs) displaying ILTV glycoproteins B (gB) or G (gG) on their surface. The main focus of this pilot study was to determine the tolerability of VLPs delivered in ovo and intramuscularly (i.m.) into chickens and to investigate the nature of the immune response elicited. The study revealed that the new vaccines were well tolerated in hybrid layer chicks independent of the administration method (in ovo or i.m.). Upon in ovo injection, vaccination with VLP-gG led to an antibody response, while a cellular immune response in VLP-gB-immunized chickens was hardly detectable. Since the administration of VLPs had no visible side effects in vivo and was shown to elicit an antibody-based immune response, we anticipate that VLPs will become a valuable platform for the development of new safe vaccines for poultry.

DOI: <https://doi.org/10.1099/jgv.0.001272>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-184866>

Journal Article

Published Version



The following work is licensed under a Creative Commons: Attribution 4.0 International (CC BY 4.0) License.

Originally published at:

Schädler, Julia; Sigrist, Brigitte; Meier, Simone Martina; Albini, Sarah; Wolfrum, Nina (2019). Virus-like particles in a new vaccination approach against infectious laryngotracheitis. *Journal of General Virology*, 100(6):1013-1026.

DOI: <https://doi.org/10.1099/jgv.0.001272>

Virus-like particles in a new vaccination approach against infectious laryngotracheitis

Julia Schädler, Brigitte Sigrist, Simone M. Meier, Sarah Albini and Nina Wolfrum*

Abstract

Gallid alphaherpesvirus 1 (syn. infectious laryngotracheitis virus; ILTV) is the causative agent of infectious laryngotracheitis, a respiratory disease of chickens causing substantial economic losses in the poultry industry every year. Currently, the most efficient way to achieve protection against infection is immunization with live-attenuated vaccines. However, this vaccination strategy entails the risk of generating new pathogenic viruses resulting from spontaneous mutations or from recombination with field strains. This work presents a new approach based on virus-like particles (VLPs) displaying ILTV glycoproteins B (gB) or G (gG) on their surface. The main focus of this pilot study was to determine the tolerability of V

LPs delivered *in ovo* and intramuscularly (i.m.) into chickens and to investigate the nature of the immune response elicited. The study revealed that the new vaccines were well tolerated in hybrid layer chicks independent of the administration method (*in ovo* or i.m.). Upon *in ovo* injection, vaccination with VLP-gG led to an antibody response, while a cellular immune response in VLP-gB-immunized chickens was hardly detectable. Since the administration of VLPs had no visible side effects *in vivo* and was shown to elicit an antibody-based immune response, we anticipate that VLPs will become a valuable platform for the development of new safe vaccines for poultry.

INTRODUCTION

Infectious laryngotracheitis (ILT) is a worldwide respiratory disease of chickens. The causative agent is the *Gallid alphaherpesvirus 1* also known as infectious laryngotracheitis virus (ILTV), which mainly infects chickens, but has also been detected in pheasants, peafowls and turkeys [1–3]. The clinical signs caused by ILTV range from reduced general conditions with mild nasal discharge, conjunctivitis and tracheitis, to serious clinical conditions with discharge of bloody mucus caused by severe laryngotracheitis, leading to dyspnoea and eventually to death by suffocation [4]. Depending on the virus strain, mortality among infected chickens varies from 10 to 70 %. Improvement of safety measures to prevent ILTV infections in chicken farms is urgently needed in order to address animal welfare issues and to reduce high annual economic loss. The latter is mainly due to milder forms of ILT resulting in egg drop in laying hens and decreased weight gain in broilers [4].

Currently, several vaccines are available. The most efficient ones are based on live attenuated viruses of tissue culture (TCO) or of chicken embryo origin (CEO). Despite providing robust protection in most instances, these vaccines bear the risk of reversion or recombination with other vaccine or field strains of ILTV, potentially resulting in novel and more virulent variants [5–7]. Recently, vectored vaccines were developed based on either the fowlpox virus or the herpesvirus of turkey, engineered to express ILTV glycoprotein B (gB) and U_L32 (CEVA, vectormune FP LT) or glycoproteins I (gI) and D (gD) (Intervet/Merck Animal Health, INNOVAX-ILT), respectively [8–11]. Because these vectored vaccines are based on low-pathogenicity or apathogenic chicken viruses, there is no risk of reversion or recombination to virulent variants. Nonetheless, these engineered vaccines are replication competent, and thus retain the potential of accumulating mutations, possibly leading to undesired effects. Furthermore, vectored vaccines reduce clinical signs, but are not able to suppress replication of challenge virus in the trachea of experimentally infected chickens [12]. Other approaches use affinity purified

Received 13 December 2018; Accepted 17 April 2019; Published 17 May 2019

Author affiliations: *National Reference Centre for Poultry and Rabbit Diseases (NRGK), Institute for Food Safety and Hygiene, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland.

***Correspondence:** Nina Wolfrum, nina.wolfrum@uzh.ch

Keywords: infectious laryngotracheitis (ILT); vaccination; virus-like particle (VLP); cellular and humoral immune response; chicken.

Abbreviations: APC, antigen-presenting cell; CAM, chorioallantoic membrane; CEO, chicken embryo origin; ChIL, chicken interleukin; DPH, days post-hatch; ED, embryonic day; gB, glycoprotein B; gD, glycoprotein D; ILT, infectious laryngotracheitis; ILTV, infectious laryngotracheitis virus; LMH, leghorn male hepatoma; MLV, murine leukaemia virus; ND, Newcastle disease; p.h., post-hatch; TCO, tissue culture origin; VLP, virus-like particle.

001272 © 2019 The Authors

This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

gB or bicistronic plasmid vectors expressing gB and chicken interleukin 18 (ChIL-18) [13, 14]. Even though the published results look promising, no further developments have been reported to date.

Another possibility, not yet applied in ILT vaccination strategies, is the engineering of virus-like particles (VLPs). Membrane-enveloped VLPs can be specifically modified to present heterologous antigens in high densities on their surface. In contrast to the aforementioned vaccine strategies, VLPs are not infectious, do not replicate and do not contain any genetic material. VLPs are thus a very safe vaccine platform. In addition, VLPs have the potential to stimulate an immune response on various levels. Due to their origin, they resemble the highly organized and repetitive symmetry of the virus from which they are derived. These features are important for efficient activation of B cells [15] and allow efficient binding of natural IgM antibodies, which in turn activate the classic complement cascade [16]. Moreover, VLPs are efficiently taken up by antigen-presenting cells (APCs). VLP shape, surface charge, hydrophobicity and hydrophilicity, but also their size (20–100 nm), mediate improved uptake into APCs as compared to smaller (<10 nm) affinity-purified proteins used as subunit vaccines [17]. Soluble proteins are inefficiently taken up by APCs and are usually only presented on MHC class II molecules leading to the activation of CD4⁺ T cells. In contrast, VLPs are degraded into peptides in APCs and can be cross-presented on MHC class I and on MHC class II molecules, thus resulting not only in the stimulation of CD4⁺ T cells but also CD8⁺ T cells [18]. In summary, VLPs are a powerful tool to stimulate the innate immune system as well as a humoral and cellular immune response.

In this report, VLPs based on the murine leukaemia virus (MLV) were engineered to present either ILTV gB or ILTV gG on their surfaces. Among herpesviruses, gB is highly conserved [19] and was shown to elicit humoral as well as cell-mediated immunity [20, 21]. ILTV gB has already been tested in various

vaccination approaches and been shown to confer protective immunity [12–14, 22–24]. Because cellular immunity is of particular importance to combat ILTV infection [25–29], VLPs-gB were further modified by maleylation. Maleylated proteins are directed to scavenger receptors present on APCs, and thus are able to induce a more dominant cellular immune response, which in turn leads to decreased levels of IgM and IgG [30–32]. To further boost the cell-mediated immunity in vaccinated chickens, either plasmids mediating expression of ChIL-18 were administered together with the VLPs, or in a second approach, VLPs displaying ILTV gG were administered together with ChIL-2. In the latter, it was anticipated to provoke a humoral immune response to counteract gG. This is important, because gG initiates an immune evasion mechanism by directing the cell-mediated immune response toward an antibody-mediated immune response [33, 34].

In this pilot study, VLPs were used for the first time as vaccines against ILTV in layer chickens. Thus, the main focus was put on examining the tolerability of the VLPs in chickens upon different inoculation methods [*in ovo* or intramuscular (i.m.)]. For animal welfare considerations, no challenge experiments were performed at this stage. However, immune responses upon vaccination were measured and shed light on the mechanisms mediating potential protection against infection with ILTV.

METHODS

Plasmid construction

For the construction of pDisplay (Invitrogen, Thermo Fisher Scientific)-based expression vectors, two different ILTV glycoprotein regions were chosen: amino acids (aa) 2 to 700 from gB and aa 30 to 293 from gG as illustrated in Fig. 1. Cloning of the gB expression construct (pDgB700) was outsourced to GeneArt Gene Synthesis Services (Thermo Fisher). To generate pDgΔ1–29, an 848 bp DNA fragment

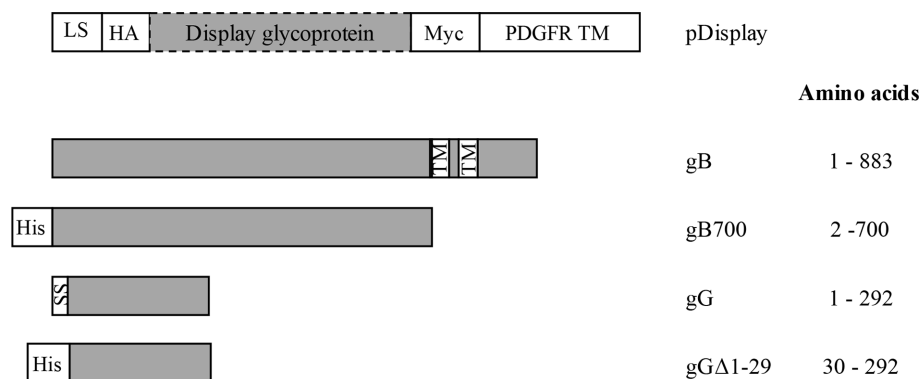


Fig. 1. Schematic illustration of the display constructs and the respective coding regions of the ILTV glycoprotein variants. The pDisplay expression plasmid encodes for the leader sequence (LS), HA and Myc tags and the transmembrane domain of the PDGF receptor (PDGFR TM). Below is a scheme of the wild type proteins (gB, gG) and of the coding regions that were inserted into pDisplay (gB700, gΔ1–29), both of which contained a His tag. Respective amino acid numbers are indicated. TM, transmembrane domain; SS, signal sequence.

Table 1. Oligonucleotides used for cloning

Bold type: restriction sites (*Bgl*II, *Pst*I, *Eco*RI, *Xho*I); underlined: 6x His-tag.

Oligo name	Oligo sequence 5'→3'
<i>gG_Bgl</i> (+)	GTAGAGATCT <u>CACCATCATCATCACCA</u> CGCTAGCGCACTGGACGGGCTCGAGTC
<i>gG_Pst</i> (-)	GACTCTGCAGCCTTCCCTCGATCTGCTGGAGCGTAGAGGGCGGTGAAAAG
<i>ChIL-2</i> (+) ₂	ATGATGTGCAAAGTACTGATCTTTGGCTGTATTTCCGGTAGCAATGC
<i>ChIL-2</i> (-) ₂	TTAATGGTGATGGTGATGGTGTTTTTCAGATATCTCACAAAGTTGG
<i>ChIL-18</i> (+) ₂	ATGAGCTGTGAAGAGATCGCAGTGTGTGCAGTACGGCTTAGAG
<i>ChIL-18</i> (-) ₂	TCAATGGTGATGGTGATGGTGTAGGTTGTGCCTTTTCATTATG
<i>EcoChIL-2</i> (+)	GCAAAGAATTCATGATGTGCAAAGTACTG
<i>EcoChIL-2</i> (-)	GGAGTGAATTCCTTAATGGTGATGGTGATG
<i>XhoChIL-18</i> (+)	AATTCCTCGAGATGAGCTGTGAAGAGATCGC
<i>XhoChIL15.18</i> (-)	AATTCCTCGAGTTAATGGTGATGGTGATG
<i>gag</i> (+)	ATGGGCCAGACTGTTACCA
<i>gag</i> (-)	CTAGTCATCTAGGGTCAGGAG

encompassing the respective part of *gG* was amplified using PCR and oligonucleotides *gG_Bgl*(+) and *gG_Pst*(-). ILTV DNA (ILTV strain 489; a kind gift from W. Fuchs and T. Mettenleiter [35]) served as template.

For construction of *ChIL-2* and *ChIL-18* expression plasmids, the respective sequences were amplified using oligonucleotides *ChIL-2*(+), *ChIL-2*(-)₂, *ChIL-18*(+)₂ and *ChIL-18*(-)₂. Chicken spleen cDNA (Amsbio) served as template. The respective amplicons were inserted into the *pcDNA3_3* TOPO vector (Invitrogen, Thermo Fisher) downstream of the cytomegalovirus (CMV) promoter resulting in constructs *pcDNA-ChIL-2-His* and *pcDNA-ChIL-18-His*. Using these two plasmids as templates, *ChIL-2* and *ChIL-18* encoding sequences were amplified using oligonucleotides *EcoChIL-2*(+), *EcoChIL-2*(-), *XhoChIL-18* and *XhoChIL15.18*(-). The amplicons were ligated into the *pCAGGS* vector (kind gift from J. Miyazaki [36]) resulting in *pCAGGS-ChIL-2-His* and *pCAGGS-ChIL-18-His*. Plasmids were isolated from bacteria using the GenElute HP Endotoxin-Free Plasmid Kit (Sigma Aldrich). Endotoxin levels were determined using the Toxin-Sensor Chromogenic LAL Endotoxin Assay Kit (GenScript).

The MLV *gag* expression construct *pcGag* was constructed by amplifying the 1617 bp *gag* encoding sequence using oligonucleotides *gag*(+) and *gag*(-) and subsequent insertion into the *pcDNA3_3* TOPO vector (Invitrogen, Thermo Fisher Scientific). The plasmid *pHit60* [37] served as a template. All primers were synthesized by Microsynth (sequences are shown in Table 1).

Gene transcription of *ChIL-2* and *ChIL-18*

To detect *ChIL-2* and *ChIL-18*, RNA was isolated from chicken cells [leghorn male hepatoma (LMH), Cell Lines Service] 48 h after transfection or from blood and organ samples from chicks using the NucleoSpin RNA Kit (Macherey Nagel).

RNA served as template for real-time RT-PCR performed as previously described by Kaiser *et al.* (*ChIL-2*) and Kogut *et al.* (*ChIL-18*) [38, 39]. In parallel, chicken GAPDH was amplified and used for normalization (*avGAPDH_for* GGAGTCAACGGATTGGCC, *avGAPDH_rev* TTTGC-CAGAGAGGACGGC, *avGAPDH_probe* FAM-TATTGGCC GCCTGGTCACCAGG-TAMRA). In brief, the reaction mixture consisted of 12.5 µl QuantiTect Probe RT-PCR Master Mix (Qiagen), 5 µl isolated RNA, 400 nM oligonucleotides, 10 nM probe and 0.25 µl QuantiTect RT Mix in a total volume of 25 µl. Amplification and detection were performed using the 7500 Fast Real-Time PCR System (Thermo Fisher Scientific) with the following cycle conditions: 50 °C for 30 min, 95 °C for 5 min, and 40 cycles of 95 °C for 20 s, 60 °C for 1 min and 72 °C for 1 min.

Production of ILTV *gB*- and ILTV *gG*-displaying VLPs

LMH cells were cultivated in 10 cm dishes in Waymouth's medium supplemented with 10 % FBS at 38 °C and 5 % CO₂. When cultured cells reached appropriate confluency, co-transfection with *pcGag* (7 µg per dish) and *pDgB700* (8 µg per dish) or *pDgΔ1–29* (8 µg per dish), respectively, was conducted under serum-free conditions employing TransIT–2020 (Mirus) according to the manufacturer's instructions. The VLP-containing cell culture supernatants were harvested 48 h later and subsequently passed through a 0.45 µm pore-size polyethersulfone filter (Sarstedt). VLPs were concentrated by ultracentrifugation (*R*_{av} 150 000 g, 3 h, 4 °C) through a 20 % sucrose cushion. The pelleted VLPs were resuspended in an appropriate amount of PBS.

Maleylation of VLP-*gB*

VLPs displaying ILTV *gB* were further modified by adding maleyl groups as previously described by Butler and Hartley

[40]. In brief, VLPs were dissolved in carbonate-bicarbonate buffer (CB; 0.1 M Na_2CO_3 , 0.1 M NaHCO_3 , pH 8.5) and maleic anhydride was added under constant monitoring of pH values. If necessary, Na_2CO_3 was added to stabilize the pH at 8.5–9.0. To detect successful maleylation, the free amino groups were determined as reported by Habeeb [41]. In brief, 100 μl of serial two-fold dilutions of the maleylated VLP-gB (mVLP-gB) and controls (non-maleylated VLP-gB, maleylated and non-maleylated BSA) were transferred to a 96-well plate. Subsequently, 60 μl of 0.05 % picrylsulfonic acid (in 0.1 M NaHCO_3) was added and incubated for 2 h at 37 °C. Reactions were stopped by adding 50 μl SDS (10 %) and 25 μl HCl (1 M) and absorbance was measured at 335 nm using the Epoch2 microplate reader (Biotek).

Electron microscopy

Concentrated VLPs were adsorbed to parlodion/carbon-coated copper grids for 2 min. Grids were rinsed four times with PBS containing 10 % goat serum and subsequently incubated for 30 min at room temperature with the anti-Myc. A7 antibody (Pierce, Thermo Fisher Scientific) diluted 1 : 50 in PBS containing 1 % goat serum. Grids were rinsed five times with PBS containing 10 % goat serum and incubated for 30 min at room temperature with 10 nm diameter gold-conjugated anti-mouse IgG (Sigma Aldrich, Merck) diluted 1 : 50 in PBS containing 1 % goat serum followed by five washes with PBS and four washes with double distilled H_2O . For the additional negative stain, grids were treated with 3 % uranyl acetate acid for a maximum of 20 s. Imaging was done using a TEM10 microscope (Zeiss) at 85 kV.

Immunofluorescence microscopy

LMH cells were grown on glass cover slips in 24-well plates. Then, 48 h after transfection with pcGag and pDgB and pDgG, respectively, cells were transferred and incubated at 4 °C for 30 min with an anti-His antibody (SignalChem) diluted 1:300 in RPMI supplemented with 2 % BSA. Cells were washed and fixated with 3 % paraformaldehyde. After washing three times with PBS, coverslips were incubated for 45 min at room temperature with an Alexa Fluor 488-coupled secondary antibody (Thermo Fisher Scientific) diluted 1:1000 in PBS containing 10 % goat serum and DAPI. After washing three times with PBS, coverslips were mounted on glass slides. Microscopic analysis was performed using an Olympus IX81 inverted epifluorescence microscope.

Western blot analysis

Transfected LMH cells were lysed with RIPA buffer 48 h after transfection and protein concentration was determined employing a BCA protein assay kit (Pierce, Thermo Fisher Scientific). Same amounts of cell lysates and concentrated VLPs were loaded onto SDS gels containing 10 % polyacrylamide. A nitrocellulose membrane was used for blotting. Proteins gB and gG were detected using an anti-HA antibody (clone16B12; Covance) and Gag was detected employing a polyclonal anti-MLV-p30 antibody (Icosagen). HRP-coupled secondary antibodies directed against rabbit

IgG (Cell Signaling Technology) and mouse IgG (Santa Cruz Biotechnology), respectively, were used for visualization together with Clarity Western ECL Substrate (BioRad) and a ChemiDoc Imager (BioRad).

Protein expression and purification

LMH cells were cultivated in 10 cm dishes in Waymouth's medium supplemented with 10 % FBS. Cells were transfected with pDgB or pDgG (each 15 μg per dish) using TransIT-2020 (Mirus) according to the manufacturer's instructions. HA-tagged gB or gG were purified employing the HA-tagged protein purification kit (MBL International Corporation). Cell lysates, samples from washing steps and the finally eluted protein were separated on an SDS gel containing 10 % polyacrylamide. A nitrocellulose membrane was used for blotting and was subsequently stained with colloidal gold (BioRad) to assess the purity of the recombinant protein preparations. Protein concentration was determined with the BCA protein assay kit (Pierce, Thermo Fisher Scientific).

Enzyme-linked immunosorbent assay

To quantify the displayed ILTV antigens, a 96-well plate was coated with serial dilutions of VLP-gB and VLP-gG, respectively, in coating buffer (CB; 50 mM NaHCO_3 , pH 9.6). Serial dilutions of purified gB and gG were used as references. The plate was incubated for 1 h at room temperature followed by an overnight incubation at 4 °C. The plate was washed using washing buffer (PBS, 0.1 % Tween 20) and incubated for 1 h at room temperature with blocking buffer I (BBI; PBS, 5 % BSA, 0.1 % Tween 20). Subsequently, the anti-Myc.A7 antibody (Pierce, Thermo Fisher Scientific) diluted 1 : 500 in BBI was added to the wells and incubated at room temperature for 1 h. After thorough washing three times, an anti-mouse HRP antibody (Santa Cruz Biotechnology) diluted 1 : 1000 in BBI was added and incubated for 1 h at room temperature. Three washing steps were performed and TMB reagent (GenScript) was added for the detection of bound HRP-coupled antibodies and incubated for 30 min at room temperature. The reactions were stopped by addition of 2 N H_2SO_4 and absorbance was measured at 450 nm using the Epoch2 microplate reader (Biotek).

To detect gG-specific antibodies in chicken serum, 96-well plates were coated with 800 ng purified gG in coating buffer. After an overnight incubation at 4 °C, plates were washed using wash buffer II (0.5 M NaCl in PBS, pH 7.2, 0.05 % Tween 20) and incubated for 2 h at 37 °C employing blocking buffer II (BBII; PBS, 1 % BSA, 0.1 % Tween 20). All sera were diluted 1 : 100 in dilution buffer (wash buffer II, 1 % BSA), added to the plate and incubated for 1 h at room temperature. Plates were washed four times and incubated for 1 h at room temperature using an HRP-coupled anti-ChIgY antibody (ProSci) diluted 1 : 5000 in dilution buffer. Plates were washed four times and TMB reagent was added for the detection of bound HRP-coupled antibodies and incubated for 30 min at room temperature. The reactions were stopped by addition of 1 M H_2SO_4 and absorbance was measured at 450 nm using the Epoch2 microplate reader (Biotek).

Table 2. Experimental design and treatments of the two study groups: gG and gB

<i>In ovo</i> (ED 18)				14 DPH	
	Group	Treatment	Amount*	Treatment	Amount*
Study group	gG	I	PBS	PBS	100 µl
		II	pcDNA-ChIL-2-His	pcDNA-ChIL-2-His	60 µg/100 µl
		III	pcDNA-ChIL-2-His VLP-gG	pcDNA-ChIL-2-His VLP-gG	60 µg 300 µg/100 µl
	gB	IV	–	PBS	100 µl
		V	–	pcDNA-ChIL-18-His	60 µg/100 µl
		VI	–	pcDNA-ChIL-18-His mVLP-gB	60 µg 250 µg/100 µl

*Amount per individual vaccination.

DPH, days post hatch; ED, embryonation day.

Animal experiments

For this study, fertilized eggs from Lohmann LSL-Classic laying hens (department's own flock) were used. The parental animals were purchased from a Swiss poultry breeding company, and thus had been vaccinated against Marek's disease, infectious bronchitis, infectious bursitis and avian encephalomyelitis. Commercial layers in Switzerland are also free from Newcastle disease (ND), ILT, *Salmonella* Enteritidis, pox, egg drop syndrome 1976, infectious coryza, *Mycoplasma gallisepticum* and *Mycoplasma synoviae*. The chickens were tested to be free from *Salmonella* and ILTV. Because commercial laying hens are the main target for ILTV vaccination, it was decided to use this common chicken hybrid.

Fertilized eggs were incubated and at embryonic day (ED) 18 randomly divided into three groups as shown in Table 2. Groups I to III comprised the study group gG and were vaccinated *in ovo*. Four eggs were injected with PBS (group I), seven eggs received plasmid pcDNA-ChIL-2-His [in PBS, DMSO 50 % (v/v), incomplete Freud's adjuvant 25 % (v/v); group II] and seven eggs were injected with plasmid pcDNA-ChIL-2-His [in PBS, DMSO 50 % (v/v), incomplete Freud's adjuvant, 25 % (v/v)] and VLP-gG (group III). VLPs were injected into the amniotic cavity whereas plasmids were administered on top of the chorioallantoic membrane (CAM). One chick in group II did not hatch. To obtain a complete data set, this treatment was repeated in parallel with study group gB as shown in Table 2. Upon hatching, chicks were individually marked with leg bands and transferred to chicken isolators (TS Group Holland). On day 14 post-hatch (p.h.) chicks of groups I to III received a respective boost of PBS or plasmid pcDNA-ChIL-2-His or plasmid pcDNA-ChIL-2-His and VLP-gG, respectively, into the musculus iliobtibialis lateralis.

Groups IV to VI comprised the study group gB without *in ovo* vaccination. Fertilized eggs were hatched, and chicks were transferred to chicken isolators, and randomly divided into three groups on day 14 p.h.: four chicks received injections of PBS (group IV), seven chicks received plasmid pcDNA-ChIL-18-His (group V), and seven chicks received plasmid

pcDNA-ChIL-18-His and mVLP-gB into the musculus iliobtibialis lateralis (group VI).

Immediately after hatching, animals were monitored every morning for signs of fitness. This visual examination was done by the same two people experienced in poultry health and behaviour in accordance with the recommendations of the Federation for Laboratory Animal Science Associations (FELASA) working group [42]. Three days post-hatch (DPH) and 2 days post-vaccination, animals were monitored twice a day (morning and afternoon). According to the observations, the vitality score was determined to enable an objective assessment of the animals' wellbeing. Vitality score criteria were as follows: 0, normal; 1, for each of the following clinical signs: closed eyelids, ruffled feathers, increased breathing, separation from the group, limping. Scores were added and interpreted as: 0, normal; 1, mild pain or distress; ≥ 2 , severe pain or distress (with euthanasia upon inspection). Weight was determined every 3–4 days, and in the same intervals blood samples were taken from the wing vein starting from day 8 p.h. (gG, groups I, II, III) or day 12 p.h. (gB, groups IV, V, VI). At the age of 35 days, chicks were euthanized and dissected. Relative weight gain was determined at the end of every week at 8, 15, 22, 29 and 35 DPH by calculating the difference between the average weight per group of the current and the past week. The value of the respective past week was set as a reference to a value of 100 %.

Isolation of spleen lymphocytes

Spleen cells were isolated by removing the capsule and passing the spleen through a 70 µm pore size cell strainer (Fisher Scientific) into isolation medium (IM; RPMI 1640, 2 % FBS). The single cell suspension was transferred onto Histopaque 1077 columns (Sigma Aldrich, Merck) and centrifuged for 20 min at 600 g at room temperature. Cells at the interface were collected in PBS and centrifuged again for 10 min at 300 g. After repeating the last step, spleen lymphocytes were collected in cultivation medium (CM; RPMI 1640, 10 % FBS,

50 μ M beta-mercaptoethanol, 100 U ml⁻¹ penicillin/streptomycin), ready for further processing.

Enzyme-linked immunospot assay

Ninety-six-well filter plates (MAIPS4510, Merck Millipore, Merck) were pre-wetted with 35 % (v/v) ethanol for 30 s, followed by three washing steps with H₂O, PBS and coating buffer (CB; 50 mM NaHCO₃, pH 9.6), respectively. An anti-ChINF- γ antibody (Ch. INF- γ Cytoset, Invitrogen, Thermo Fisher Scientific) was added (0.4 μ g per well) and incubated overnight at 4 °C. Plates were rinsed twice with blocking buffer III (BBIII; RPMI 1640, 2 % FBS, 50 μ M beta-mercaptoethanol, 100 U ml⁻¹ penicillin/streptomycin) and incubated with BBIII for 1 h at 38 °C under 5 % CO₂. BBIII was discarded and 5 \times 10⁵ spleen lymphocytes in 100 μ l CM were seeded per well. Cells were incubated in the presence of either culture medium or medium supplemented with one of the following stimulating reagents to a final volume of 200 μ l per well: ionomycin (2.8 μ M) and phorbol 12-myristate 13-acetate (PMA; 0.65 μ M), purified gB (4 μ g) or ILTV strain 489 (m.o.i. 2). Cells were incubated for 48 h at 38 °C in a 5 % CO₂ atmosphere. The supernatants were discarded and plates were rinsed twice with H₂O and three times with wash solution (WS; PBS, 0.1 % Tween 20). Plates were incubated for 1 h at room temperature with a biotinylated anti-ChINF- γ antibody (Ch. INF- γ Cytoset, Invitrogen, Thermo Fisher Scientific) diluted 1 : 310 in BBII. After four rinses with WS, plates were incubated for 1 h at room temperature with streptavidin-HRP diluted 1 : 1000 in BBII. Subsequently, plates were washed five times with PBS and incubated for 40 min with Novex HRP Chromogenics Substrate (Thermo Fisher Scientific). After rinsing under running water, plates were air-dried and analysed with an AID ELISPOT reader (AID Autoimmun Diagnostika).

Fluorescence-activated cell scanning

Citrated blood samples (~500 μ l) were mixed 1 : 2 with PBS and transferred onto Histopaque 1077 columns (Sigma Aldrich, Merck) and centrifuged for 20 min at 400 g at room temperature. Cells at the interface were collected into PBS and centrifuged again for 5 min at 400 g at 4 °C. Upon suspension of the cell pellet in FACS buffer (PBS, 2 % FBS, 0.05 % NaN₃), cells were counted and aliquoted at a concentration of 5 \times 10⁵ cells/50 μ l. Cells were kept at 4 °C and were analysed for the surface expression of CD3⁺, CD4⁺ and CD8⁺, respectively. Unstained cells served as negative controls. To facilitate compensation for spectral overlap, single stains were also performed. The FITC-coupled anti-ChCD3 antibody (Abcam) was used at a final concentration of 5 μ g ml⁻¹, the PE-coupled anti-ChCD4 antibody (Acris) was used at a final concentration of 0.2 μ g ml⁻¹, and the PE-coupled anti-ChCD8

antibody (antibodies-online.com) was applied at a final concentration of 0.5 μ g ml⁻¹. Cells were incubated for 45 min at 4 °C followed by centrifugation for 5 min at 400 g at 4 °C. Cells were washed with FACS buffer and centrifuged again. Upon fixation with 1 % paraformaldehyde for 10 min and a final centrifugation, cells were resuspended in FACS buffer and filtered through a 35 μ m cell strainer into a polystyrene round bottom tube (Fisher Scientific). Samples were analysed using a FACS Canto (BD Bioscience) at the flow cytometry facility of the University of Zurich by acquiring 10 000 events in the lymphocyte gate. Data analysis was performed using the BD FACSDIVA (BD Bioscience) and the FlowJo, LLC software.

Statistical analysis

To calculate the number of animals needed to reveal significant results, the software G*Power was used. Mean values, percentages, standard deviations and ANOVA variance test (single factor) results were determined using Excel.

RESULTS

Expression of ChIL-2 and ChIL-18 in chicken LMH cells

The sequences of each chicken interleukin (ChIL-2, ChIL-18) were cloned into expression vectors containing either the CMV promoter or the CAG promoter consisting of the CMV immediate early enhancer followed by the chicken beta actin (CBA) promoter and the rabbit beta globin intron. To examine potential variations in promoter-dependent expression, the respective plasmids were transfected into LMH cells and total RNA was analysed 48 h later using real-time RT-PCR. As shown in Table 3, C_t values did not differ significantly between ChIL-2 or -18 expressed from the CMV promoter or from the CAG promoter. In all further experiments described here, the ChIL-expression plasmids containing the CMV promoter were used.

Generation and characterization of VLPs

To achieve the display of ILTV gB and gG on the surface of VLPs, respectively, the corresponding coding regions were inserted into the pDisplay vector, encoding the murine immunoglobulin chain leader sequence as well as the transmembrane domain of the platelet-derived growth factor receptor (PDGFR), thereby facilitating transport through the secretory pathway and anchoring of the respective fusion protein in the plasma membrane. For gB, the coding region for the first 700 aa was inserted, consequently depleting the transmembrane domain and the cytoplasmic tail at the C terminus. For gG, the

Table 3. Threshold cycle (C_t) values from real-time RT-PCRs performed in duplicate on total RNA extracted from transfected LMH cells

Interleukin	ChIL-2			ChIL-18		
Promoter	Mock	CMV	CAG	Mock	CMV	CAG
C _t	ND/ 30.91	15.75/15.65	15.54/15.38	27.28/27.31	14.07/14.07	14.87/14.26

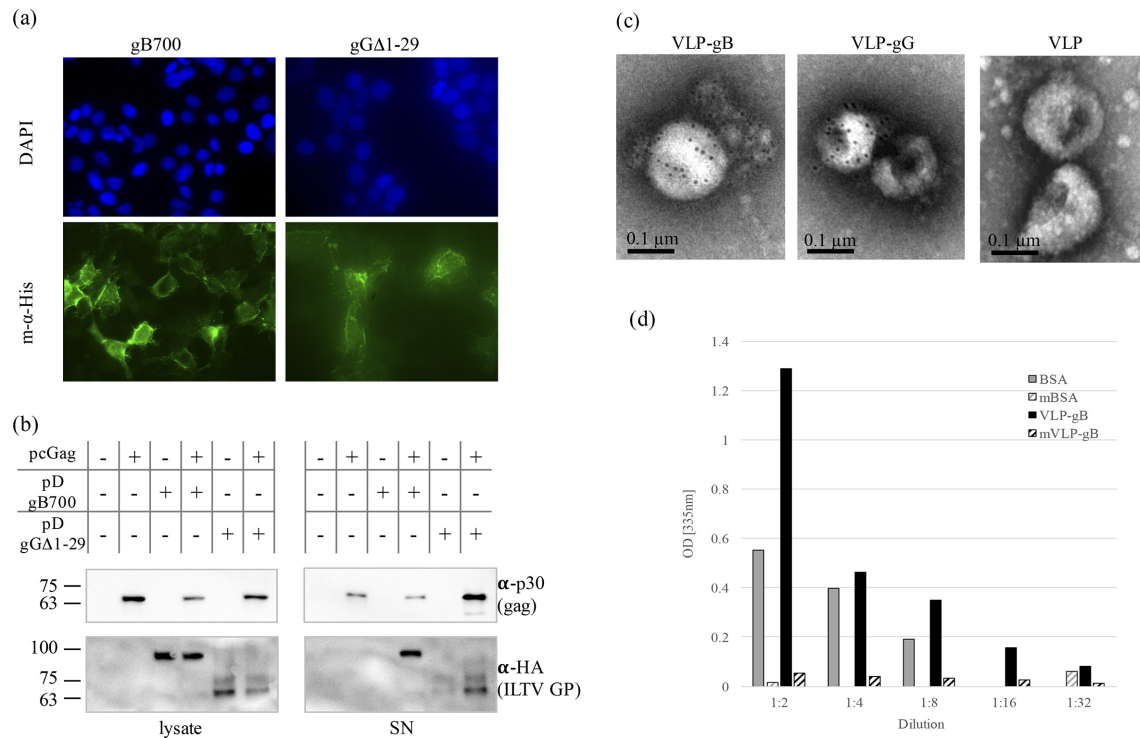


Fig. 2. ILTV glycoprotein variant expression and characterization of VLPs. (a) Surface expression of gB700 and gGA1–29 on transiently transfected LMH cells. Upper row: cell nuclei were stained with DAPI. Lower row: ILTV glycoproteins were visualized by immunofluorescence using anti-His antibodies. (b) Western blot analysis of cell lysates and supernatants of LMH cells transiently transfected with the indicated expression plasmids. MLV Gag was detected using anti-p30 antibodies. ILTV glycoproteins (GP) were detected using anti-HA antibodies. (c) Electron micrographs of VLPs displaying ILTV glycoprotein variants detected with gold-labelled anti-Myc tag antibodies. (d) Determination of free amino groups on maleylated VLP-gB (mVLP-gB) and control BSA.

N-terminal signal sequence was removed and only the coding region for amino acid positions 30 to 292 was inserted into the expression vector (Fig. 1). Besides the pDisplay-encoded HA and Myc tags, the expressed fusion proteins described here harboured a His tag, which was introduced with the oligonucleotides used to amplify the respective DNA fragments prior to insertion into the vector (compared in Table 1).

Surface expression of the resulting ILTV protein variants gB700 and gGA1–29 was verified by immunofluorescence staining of LMH cells transiently transfected with the respective expression constructs. To ensure surface staining, living cells were kept on ice during exposure to antibodies and thereafter fixed. As illustrated in Fig. 2 (a), both glycoproteins were readily detected.

To verify the expression of VLPs displaying gB700 and gGA1–29, respectively, LMH cells were either transiently transfected with the respective ILTV glycoprotein variant expression plasmids only or co-transfected together with pcGag. As illustrated in Fig. 2b, lysates and concentrated supernatants of transfected cells were subjected to Western blotting analysis. Since pcGag encodes only the MLV Gag protein and not the viral protease, the anti-p30 antibody detected the Gag precursor protein p65 and not the capsid protein p30 in cell lysates and supernatants. Both ILTV glycoprotein variants

were detected in cell lysates independent of the co-expression of Gag. As expected, gB700 and gGA1–29 revealed approximate molecular weights of 100 and 60 kDa, respectively. In contrast, both ILTV protein variants were detected only in supernatants that also contained Gag. This indicated incorporation of the ILTV protein variants into MLV Gag-formed VLPs. This observation was further supported by electron microscopy using immunogold-labelled antibodies specific for the Myc tag present in both viral protein variants. As shown in Fig. 2c, particle structures were detected in all VLP preparations. Gold particles located at the membrane of these particles were visualized in VLP-gB and VLP-gG preparations but not in controls consisting of ‘naked’ VLPs. This further substantiated the decoration of VLPs with both ILTV protein variants.

VLPs-gB were further modified by maleylation. To measure a successful reaction, the availability of free amino groups of VLPs-gB was analysed. As a control, BSA was maleylated in parallel. The relative amount of free amino groups of unmodified proteins decreased with increasing dilution. In contrast, the relative amount of free amino groups of the maleylated proteins was constant at a very low level, indicating a successful maleylation of the proteins, as shown in Fig. 2d.

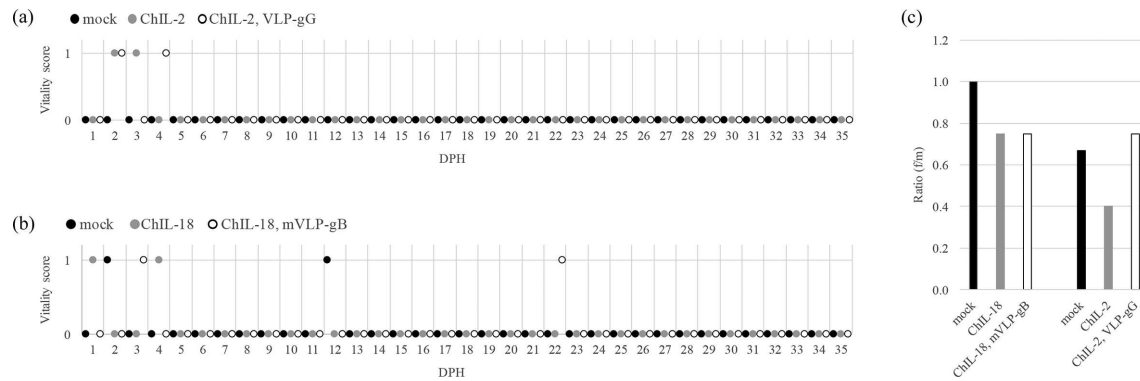


Fig. 3. (a, b) Vitality scores of vaccinated and control chicks. (a) gB group, i.m. injection on day 14. (b) gG group, *in ovo* vaccination and i.m. booster vaccination on day 14. Bold, blood sampling; underlined, day of (booster) vaccination. (c) Ratio of female to male chicks in the respective groups.

Tolerability of the vaccines

To identify potential adverse effects of the vaccine on the development and wellbeing of the chicken embryos or hatched chicks, several factors were taken into consideration, i.e. hatching rate, vitality score (indicating general health and wellbeing), sex ratio and weight gain.

The hatching rate of all *in ovo* injected individuals was 95 % (18 chicks out of 19 hatched; 18/19), including 4/4 (100 %) in the negative control group, which received only PBS (group I), 6/7 (86 %) in the group being treated with plasmids encoding ChIL-2 (group II), and 7/7 (100 %) in animals receiving plasmids expressing ChIL-2 and VLPs (group III). As depicted in Fig. 3a and b, single chicks showed mild signs of distress mainly in the first few days after hatching reflected by a vitality score of 1. However, this was not related to a particular treatment but a result of post-hatching adaptation to a new environment and food uptake. On day 12 p.h., a mock treated chick (PBS) of the gB group was rated with a score of 1 due to peculiar sleepiness (closed eyelids) probably associated with blood sampling earlier that day. No unusual behaviour was detected on the following day. Score 1 was also attributed to a ChIL-18, mVLP-gB-treated chick as breathing difficulties were observed. Examination of the chick revealed fibre originating from the bedding material stuck at the entrance of the larynx. Upon removal of the fibre, breathing normalized again. In summary, none of the clinical signs observed in the chicks could be attributed to vaccination.

The sex ratio in each group had – as expected for a layer hen line – a slight tendency towards more males as shown in Fig. 3c and as previously described [43].

Within the respective study groups, the weight of the chicks increased over time with no statistical significance between the different treatments (PBS, plasmid, plasmid and VLP) as shown in Fig. 4a, b. However and as illustrated in Fig. 4c, a difference was noted between the two study groups gB and gG concerning average weight gain per week. Although weight at hatch was comparable between all groups (on average 39 g), weight gain during the first week was much higher

for gB groups IV–VI, which almost doubling their weight compared to the gG groups, which only gained around 30 % of their initial weight. However, in week 2 the relative weight gain of the gG group reached the level of the gB group, and even exceeded them in weeks 3 and 4, leading to average weights within the gG group of 351 g that were about 10 % lower than the average weight of the gB group (393 g) after week 5.

Transcription of ChIL-2/-18 upon plasmid injection *in ovo* or i.m.

To promote a humoral or cellular immune response, plasmid encoded ChIL-2 or ChIL-18 were administered in addition to the respective VLPs. Expression of both interleukins was monitored by qRT-PCR at various time points after vaccination. No differences in ChIL-2 or ChIL-18 levels were detected in the blood (or any other investigated organ) of animals receiving plasmids expressing chicken interleukins compared to PBS-treated animals (data not shown).

Immune response to VLPs-gB

Application of ILTV gB-displaying VLPs – further modified by maleylation – and co-administration of plasmid encoded ChIL-18 aimed at inducing a cell-mediated immune response. To examine this, the IFN- γ response of spleen lymphocytes upon stimulation with different antigens was conducted. Three weeks after vaccination, at the age of 35 days, chicks were euthanized and spleens were processed for further analysis using an ELISPOT assay performed in triplicate. The average number of spots is depicted in Fig. 5a. Lymphocytes from all animal groups treated with PBS, ChIL-18, ChIL-18 and mVLP-gB, respectively, responded to stimulation with ionomycin and PMA, used as a positive control. Nevertheless, no reaction was detected upon stimulation of the lymphocytes with ILTV or gB alone.

The IFN- γ response of spleen lymphocytes cannot be monitored at multiple time points in one individual animal. Thus, the ratio of CD4⁺ to CD8⁺ T lymphocytes was determined

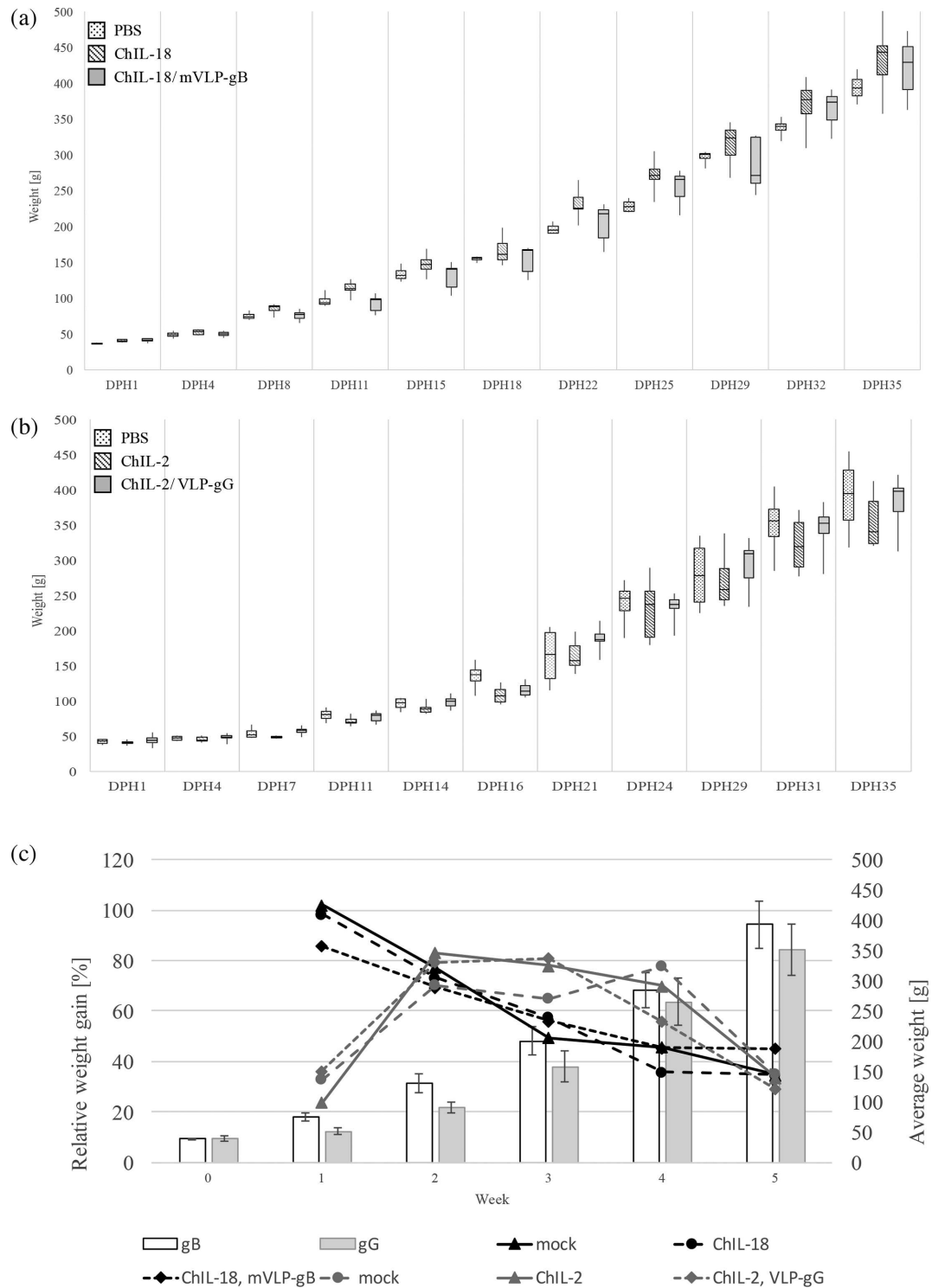


Fig. 4. Weight development of chicks during the experimental period. (a) Weight development of chicks of the study group gB (b) and study group gG (c). Relative weight gain per week (lines) per individual treatment and average weight (bars) of the whole group.

as an additional indicator of the cellular immune response. These values are depicted in Fig. 5b as box plots as well as the average of the three treatment groups including a corresponding trend line. A treatment-independent increase in

the ratio of CD4⁺ to CD8⁺ cells 2 days after vaccination (DPH 16) was detected. At later time points, a decrease of the CD4⁺ to CD8⁺ ratio was observed. This decrease was slightly more pronounced in chicks vaccinated with ChIL-18 and mVLP-18.

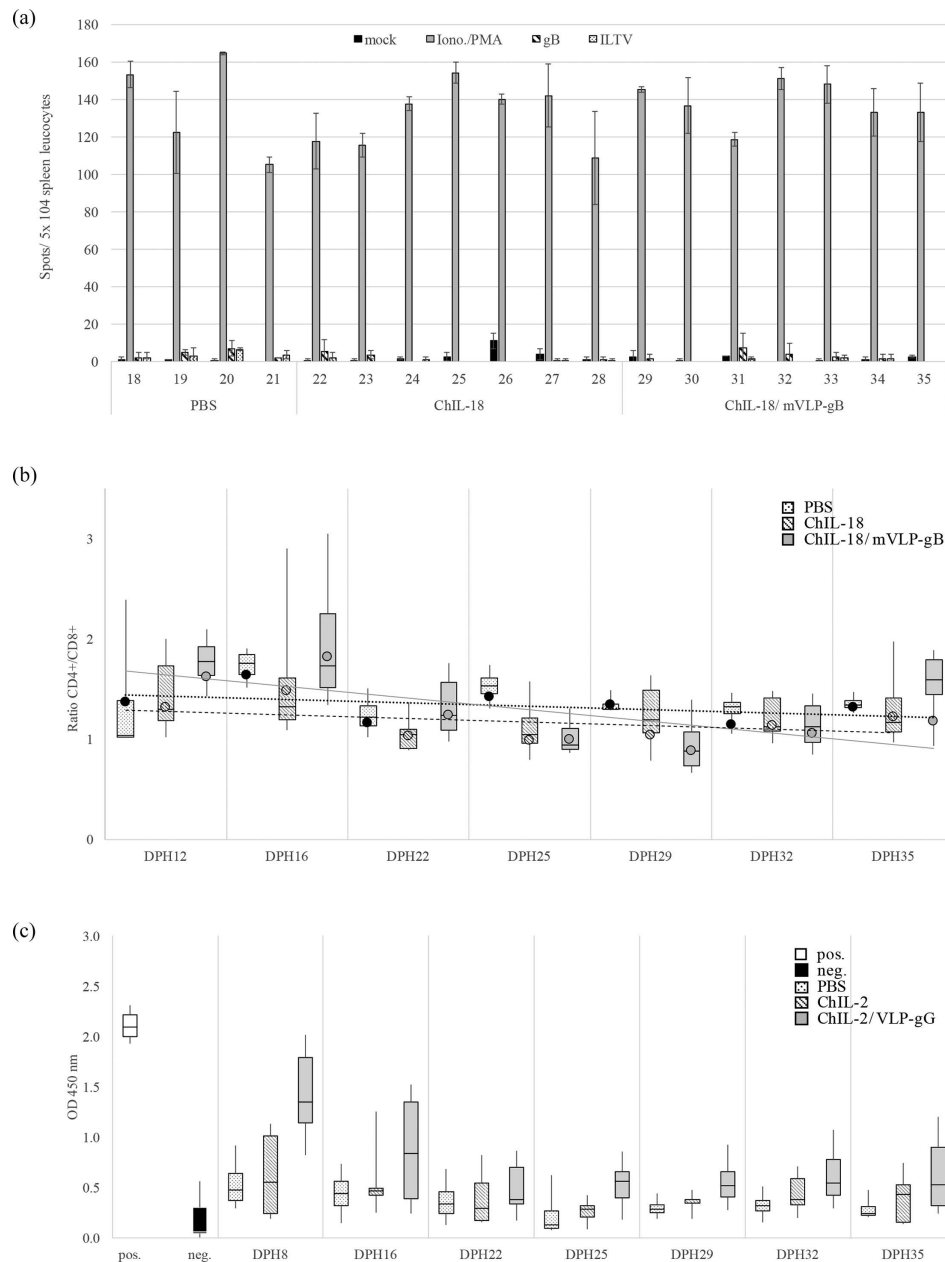


Fig. 5. Immune response. (a) IFN- γ ELISPOT responses to ILTV antigens. The total magnitude of IFN- γ ELISPOT responses is given for individual chicks. The ELISPOT assay was performed in triplicate. (b) Ratios of CD4⁺/CD8⁺ T cells determined by FACS. (c) Humoral response in VLP-gG-vaccinated and control chicks. The antibody response was measured at various time points after *in ovo* prime and booster vaccination. The ELISA was conducted in duplicate and repeated twice.

Immune response to VLPs-gG

Vaccination with VLP-gG and plasmid-encoded ChIL-2 aimed at provoking a humoral immune response, which should prevent – in case of an ILTV infection – the gG-induced immune evasion mechanism. ILTV gG-specific antibodies were detected using ELISA. Results are depicted as box blots in Fig. 5c. At the first time point of measurement (DPH 8) 11 days after *in ovo* vaccination, gG-specific antibodies were detected. Titres decreased on DPH 16 and reached background levels on DPH 22.

DISCUSSION

The present pilot study describes the use of heterologous VLPs as a vaccination platform against ILTV in chickens. VLP-based poultry vaccines were mainly investigated in the context of avian influenza and ND and showed promising results such as high antigenicity and even protection from infection [44–46]. Most studies directly examine the protection that is conferred by a particular vaccine. For animal welfare reasons, this study focused first on determining the

tolerability as well as the immune responses elicited upon vaccination.

Because MLV-based VLPs are enveloped with a membrane originating from the cells utilized for production, it was decided to generate the VLPs in a chicken cell line (LMH) to avoid immune reactions directed against heterologous host cell proteins. Expression levels of the ILTV glycoprotein variant-displaying VLPs in LMH cells was comparable to the productivity in the most frequently used human producer cell line 293T (data not shown).

The *in ovo* injection had no impact on hatching rate, which is on average between 85 and 95 % upon artificial incubation [47, 48] and ranged between 86 and 100 % in this study. Neither *in ovo* administration nor i.m. injection resulted in adverse effects in chicks as rated in the vitality score. No influence on the weight at hatch was observed. Notably, the weekly weight gain was reduced in the *in ovo* vaccinated gG group compared to the gB group receiving no treatment prior to hatch. Unfortunately, we did not find earlier reports examining the effect of *in ovo* mock injections in comparison to no manipulation at all. We assumed that the observed difference was not related to the vaccine but rather a result of the injection procedure as animals in the control groups (injection of PBS and plasmid encoding ChIL-2, respectively) also revealed a delayed weight gain. Another factor that could have contributed to the delayed weight gain is the different age of the breeders at the time of egg-laying [49] as the experiments with the two groups (gG, gB) were not conducted in parallel.

Previous experiments showed that ChIL-2 was detectable in blood, thymus and liver of 20-day-old embryos 2 days after administration of ChIL-2 expression plasmids through the CAM. In the current study, expression of ChIL-2 was detected when blood was analysed on day 8 p.h. (11 days after *in ovo* administration of the ChIL-2 expression plasmids) or at later time points (DPH: 16, 22, 25, 29, 35). However, no differences were observed between the control group (PBS) and groups who received the ChIL-2 expression plasmids. This might be due to an onset of endogenously produced ChIL-2, which can occur between days 2 and 4 p.h. [50]. The same was observed for the expression of ChIL-18: no difference was detected between the control group (PBS) and chickens that received the expression plasmid when analysed 2 days after i.m. administration on day 14. This could also be due to an early onset of constitutive ChIL-18 expression already on ED 12 and an increase immediately upon hatching [51]. For future experiments, a detection method needs to be utilized enabling the differentiation between endogenous and recombinant interleukins.

Various studies have indicated a particular importance of the cellular immunity to protect against ILTV infection [25–29]. This issue was addressed in the current study by maleylation of the gB-displaying VLPs and co-administration of plasmids mediating the expression of ChIL-18. Although thymocytes in the chicken embryo respond to mitogen stimulation as early as ED 14, these cells reach their full activation capacity at an age of about 2 weeks [52]. Thus, it was decided to apply

the vaccine at a stage where it was more likely to elicit a robust cellular immune response. Nevertheless, a strong cellular immune response could not be detected. The ELISPOT assay did not reveal any IFN- γ response upon stimulation with the antigen. One potential reason for this might be the time point of sampling at 21 days post-vaccination, and thus, rather late compared to other vaccination studies describing sampling between 7 and 17 days post-vaccination [53–55]. To monitor the cellular immune response over time, the ratio of CD4⁺ to CD8⁺ T cells was determined by FACS. This ratio is an indicator for immune competence. Upon virus infection (or vaccination), cytotoxic T lymphocytes, which are largely CD8⁺, undergo massive expansion [56–58]. CD4⁺ T cells also expand, but their differentiation into subsets that possess distinct effector functions is more pronounced. The expansion phase lasts for about 2 weeks and peaks at about 1 week after antigen exposure. Thus, virus infection is usually accompanied by a decreased CD4⁺/CD8⁺ T cell ratio. In healthy, non-vaccinated chickens the CD4⁺/CD8⁺ T cell ratio remains relatively constant even at different ages, but it can vary between different chicken lines and housing conditions. For specific pathogen-free chickens, lower ratios (between 0.74 and 0.94) were found compared to different laying hen lines kept under regular housing conditions (Dekalb Delta hybrid: 1.2; outbred white leghorn chicken: 3.25) [59, 60]. In the current study, no statistically significant difference in CD4⁺/CD8⁺ ratio over time for control treated animals (PBS: 1.34 \pm 0.17, ChIL-18: 1.17 \pm 0.18) was observed. This is in accordance with previous studies reporting similar findings for chickens kept in regular environments. In contrast, the vaccinated chicks showed a slight trend towards an increase of CD8⁺ cells. This contradicts the results of an ILTV vaccination study published by Chen and colleagues, who detected an increase of the CD4⁺/CD8⁺ ratio and furthermore associated this increase with the conferred protection [61]. In contrast, protective vaccination against ND and fowl adenovirus, respectively, were reported to be a result of a decreased CD4⁺/CD8⁺ ratio [62, 63].

To counteract the ILTV gG-induced immune evasion, this study aimed at inducing gG-specific antibodies using *in ovo* vaccination with gG-displaying VLPs and ChIL-2 expression constructs. To further increase the immune response, a booster vaccination was administered i.m. on day 14. On the first day of sampling (DPH 8, 11 days after vaccination), increased anti-gG antibody levels were detected. This was a promising result, as ILTV gG seems to be only weakly antigenic [64]. The success of the described approach is probably due to the high density of antigens decorated on the surface of the membrane-enveloped VLPs. The elicited anti-gG antibody levels decreased over time and reached background levels on day 22 (1 week after the boost). Surprisingly, the boost did not lead to an increased or at least constant antibody titre. The detected effect on the cellular immune response was also weaker than expected and was probably a result of the comparably low amount of antigen used. Most vaccines currently administered *in ovo* are either replication competent attenuated live vaccines or vector-derived vaccines. The replication incompetence of VLPs, which is an

advantage regarding safety, might be a drawback regarding the induction of a robust immune response. Yet various studies have shown that a single *in ovo* vaccination with a replication-incompetent reagent such as outer membrane vesicles of gram-negative bacteria or antigen–antibody complexes (AACs) can induce at least an antibody response [65, 66]. Nevertheless, we propose a higher dosage for the i.m. application, which should be determined according to the actual body weight at the time of injection. The average weight of a layer chicken embryo at ED 18 is 22 g, and thus the injected amount of vaccine corresponds to 14 µg g⁻¹ body weight (bw). In contrast, the injected amount of VLP in the 14-day-old chicks was around 2–3 µg g⁻¹ bw for VLP-gB or VLP-gG respectively. Furthermore, analysis of the cellular immune response by ELISPOT should be conducted at two time points closer to the time of vaccination, e.g. 7 and 14 days post-injection as in earlier vaccination studies [53, 54].

In summary, the MLV-derived VLPs displaying ILTV glycoprotein variants were well tolerated and were able to induce a detectable immune response. Thus, this new approach could initiate the further development of ILTV vaccines and the generation of safer vaccines for poultry in general.

Funding information

The authors received no specific grant from any funding agency.

Acknowledgements

We would like to thank Karin Boucke for her valuable support with electron microscopy and the flow cytometry facility of the University of Zurich for their assistance. Furthermore, we greatly appreciated the help of Sibylle Grosjean and Jasmin Steiner and we would like to thank Jörn Stitz and Magda Nüesch-Inderbinden for proofreading the manuscript.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

Animal experiments were approved by the Veterinary Office of the Canton of Zurich under licence ZH004/17. All protocols were carried out in strict accordance with the Swiss Federal Food Safety and Veterinary Office guidelines (Animal Protection Act, Animal Protection Ordinance, Animal Experimentation Ordinance). The experiments were performed in the University of Zurich, Vetsuisse Faculty, animal house facilities approved by the Veterinary Office of the Canton of Zurich (ZH0261/70/59).

References

- Crawshaw GJ, Boycott BR. Infectious laryngotracheitis in peafowl and pheasants. *Avian Dis* 1982;26:397–401.
- Portz C, Beltrão N, Furian TQ, Júnior AB, Macagnan M *et al*. Natural infection of turkeys by infectious laryngotracheitis virus. *Vet Microbiol* 2008;131:57–64.
- Winterfield RW, So IG. Susceptibility of turkeys to infectious laryngotracheitis. *Avian Dis* 1968;12:191–202.
- García M, Spatz S, Guy JS, Roepke WJ. Infectious Laryngotracheitis. In: Swayne DE (editor). *Diseases of Poultry*, 13th ed. John Wiley & Sons, Inc; 2017. pp. 161–179.
- Lee S-W, Markham PF, Coppo MJC, Legione AR, Markham JF *et al*. Attenuated vaccines can recombine to form virulent field viruses. *Science* 2012;337:188.
- Agnew-Crumpton R, Vaz PK, Devlin JM, O'Rourke D, Blacker-Smith HP *et al*. Spread of the newly emerging infectious laryngotracheitis viruses in Australia. *Infect Genet Evol.* Elsevier B.V.; 2016;43:67–73.
- Blacker HP, Kirkpatrick NC, Rubite A, O'Rourke D, Noormohammadi AH. Epidemiology of recent outbreaks of infectious laryngotracheitis in poultry in Australia. *Aust Vet J* 2011;89:89–94.
- Davison S, Gingerich EN, Casavant S, Eckroade RJ. Evaluation of the efficacy of a live fowlpox-vectored infectious laryngotracheitis/avian encephalomyelitis vaccine against ILT viral challenge. *Avian Dis* 2006;50:50–54.
- Tong GZ, Zhang SJ, Wang L, Qiu HJ, Wang YF *et al*. Protection of chickens from infectious laryngotracheitis with a recombinant fowlpox virus expressing glycoprotein B of infectious laryngotracheitis virus. *Avian Pathol* 2001;30:143–148.
- Gimeno IM, Cortes AL, Guy JS, Turpin E, Williams C. Replication of recombinant herpesvirus of turkey expressing genes of infectious laryngotracheitis virus in specific pathogen free and broiler chickens following *in ovo* and subcutaneous vaccination. *Avian Pathol* 2011;40:395–403.
- Esaki M, Noland L, Eddins T, Godoy A, Saeki S *et al*. Safety and efficacy of a turkey herpesvirus vector laryngotracheitis vaccine for chickens. *Avian Dis* 2013;57:192–198.
- Johnson DI, Vagnozzi A, Dorea F, Riblet SM, Mundt A *et al*. Protection against infectious laryngotracheitis by *in ovo* vaccination with commercially available viral vector recombinant vaccines. *Avian Dis* 2010;54:1251–1259.
- York JJ, Fahey KJ. Vaccination with affinity-purified glycoproteins protects chickens against infectious laryngotracheitis herpesvirus. *Avian Pathol* 1991;20:693–704.
- Chen H-Y, Zhao L, Wei Z-Y, Cui B-A, Wang Z-Y *et al*. Enhancement of the immunogenicity of an infectious laryngotracheitis virus DNA vaccine by a bicistronic plasmid encoding glycoprotein B and interleukin-18. *Antiviral Res.* Elsevier B.V.; 2010;87:235–241.
- Bachmann MF, Rohrer UH, Kündig TM, Bürki K, Hengartner H *et al*. The influence of antigen organization on B cell responsiveness. *Science* 1993;262:1448–1451.
- Ehrenstein MR, Notley CA. The importance of natural IgM: scavenger, protector and regulator. *Nat Rev Immunol.* Nature Publishing Group; 2010;10:778–786.
- Ahsan F, Rivas IP, Khan MA, Torres Suárez AI. Targeting to macrophages: role of physicochemical properties of particulate carriers—liposomes and microspheres—on the phagocytosis by macrophages. *J Control Release* 2002;79:29–40.
- Kovacs-Bankowski M, Clark K, Benacerraf B, Rock KL. Efficient major histocompatibility complex class I presentation of exogenous antigen upon phagocytosis by macrophages. *Proc Natl Acad Sci U S A* 1993;90:4942–4946.
- Pereira L. Function of glycoprotein B homologues of the family Herpesviridae. *Infect Agents Dis* 1994;3:9–28.
- Keller PM, Neff BJ, Ellis RW. Glycoprotein neutralization 1984;52:293–297.
- Zarling JM, Moran PA, Burke RL, Pacht C, Berman PW *et al*. Human cytotoxic T cell clones directed against herpes simplex virus-infected cells. IV. Recognition and activation by cloned glycoproteins gB and gD. *J Immunol* 1986;136:4669–4673.
- Vagnozzi A, Zavala G, Riblet SM, Mundt A, García M. Protection induced by commercially available live-attenuated and recombinant viral vector vaccines against infectious laryngotracheitis virus in broiler chickens. *Avian Pathol* 2012;41:21–31.
- Yu Q, Spatz S, Li Y, Yang J, Zhao W *et al*. Newcastle disease virus vectored infectious laryngotracheitis vaccines protect commercial broiler chickens in the presence of maternally derived antibodies. *Vaccine* 2017;35:789–795.
- Zhao W, Spatz S, Zhang Z, Wen G, Garcia M *et al*. Newcastle disease virus (NDV) recombinants expressing infectious laryngotracheitis virus (ILTV) glycoproteins gB and gD protect chickens against ILTV and NDV challenges. *J Virol* 2014;88:8397–8406.
- Coppo MJC, Hartley CA, Devlin JM. Immune responses to infectious laryngotracheitis virus. *Dev Comp Immunol.* Elsevier Ltd; 2013;41:454–462.

26. Fahey KJ, York JJ, Bagust TJ. Laryngotracheitis herpesvirus infection in the chicken. II. the adoptive transfer of resistance with immune spleen cells. *Avian Pathol* 1984;13:265–275.
27. Fahey KJ, Bagust TJ, York JJ. Laryngotracheitis herpesvirus infection in the chicken: the role of humoral antibody in immunity to a graded challenge infection. *Avian Pathol* 1983;12:505–514.
28. Fahey KJ, York JJ. The role of mucosal antibody in immunity to infectious laryngotracheitis virus in chickens. *J Gen Virol* 1990;71:2401–2405.
29. Honda T, Okamura H, Taneno A, Yamada S, Takahashi E. The role of cell-mediated immunity in chickens inoculated with the cell-associated vaccine of attenuated infectious laryngotracheitis virus. *J Vet Med Sci* 1994;56:1051–1055.
30. Abraham R, Singh N, Mukhopadhyay A, Basu SK, Bal V et al. Modulation of immunogenicity and antigenicity of proteins by maleylation to target scavenger receptors on macrophages. *J Immunol* 1995;154:1–8.
31. Haberland ME, Fogelman AM. Scavenger receptor-mediated recognition of maleyl bovine plasma albumin and the demaleylated protein in human monocyte macrophages. *Proc Natl Acad Sci U S A* 1985;82:2693–2697.
32. Vandaveer SS, Erf GF, Durdik JM. Avian T helper one/two immune response balance can be shifted toward inflammation by antigen delivery to scavenger receptors. *Poult Sci* 2001;80:172–181.
33. Devlin JM, Legione AR, Vaz PK, Lee S-W, Gilkerson JR M et al. Infectious laryngotracheitis virus viral chemokine-binding protein glycoprotein G alters transcription of key inflammatory mediators in vitro and in vivo. *J Virol* 2018;92:1–17.
34. Devlin JM, Viejo-Borbolla A, Browning GF, Noormohammadi AH, Gilkerson JR et al. Evaluation of immunological responses to a glycoprotein G deficient candidate vaccine strain of infectious laryngotracheitis virus. *Vaccine* 2010;28:1325–1332.
35. Fuchs W, Mettenleiter TC. DNA sequence and transcriptional analysis of the UL1 to UL5 gene cluster of infectious laryngotracheitis virus. *J Gen Virol* 1996;77:2221–2229.
36. Niwa H, Yamamura K, Miyazaki J, Hitoshi N, Ken-ichi Y, Jun-ichi M. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 1991;108:193–199.
37. Soneoka Y, Cannon PM, Ramsdale EE, Griffiths JC, Romano G et al. A transient three-plasmid expression system for the production of high titer retroviral vectors. *Nucl Acids Res* 1995;23:628–633.
38. Kaiser P, Rothwell L, Galyov EE, Barrow PA, Burnside J et al. Differential cytokine expression in avian cells in response to invasion by *Salmonella typhimurium*, *Salmonella enteritidis* and *Salmonella gallinarum*. *Microbiology* 2000;146:3217–3226.
39. Kogut MH, Rothwell L, Kaiser P. Priming by recombinant chicken interleukin-2 induces selective expression of IL-8 and IL-18 mRNA in chicken heterophils during receptor-mediated phagocytosis of opsonized and nonopsonized *Salmonella enterica* serovar enteritidis. *Mol Immunol* 2003;40:603–610.
40. Butler PJ, Hartley BS. Maleylation of amino groups. *Methods Enzymol* 1972;25:191–199.
41. Habeeb AF. Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Anal Biochem* 1966;14:328–336.
42. Borrens M, Girod A, Lelovas P, Morrison F, Torres YS. The reporting of clinical signs in laboratory animals 2015.
43. Klein S, Grossmann R. Primary sex ratio in fertilized chicken eggs (*Gallus Gallus domesticus*) depends on reproductive age and selection. *J Exp Zool A Ecol Genet Physiol* 2008;309A:35–46.
44. Park J-K, Lee D-H, Yuk S-S, Tseren-Ochir E-O, Kwon J-H et al. Virus-like particle vaccine confers protection against a lethal Newcastle disease virus challenge in chickens and allows a strategy of differentiating infected from vaccinated animals. *Clin Vaccine Immunol* 2014;21:360–365.
45. Noh J-Y, Park J-K, Lee D-H, Yuk S-S, Kwon J-H et al. Chimeric bivalent virus-like particle vaccine for H5N1 HpaI and nd confers protection against a lethal challenge in chickens and allows a strategy of differentiating infected from vaccinated animals (Diva). *PLoS One* 2016;11:e0162946–13.
46. Kapczynski DR, Tumpey TM, Hidayat R, Zsak A, Chrzastek K et al. Vaccination with virus-like particles containing H5 antigens from three H5N1 clades protects chickens from H5N1 and H5N8 influenza viruses. *Vaccine* 2016;34:1575–1581.
47. Weissmann A, Gottschalk J, Reitemeier S, Preisinger R, Einspanier A. In ovo-gender identification in laying hen hybrids: Effects on hatching and production performance. *Eur Poult Sci* 2014;78:1–12.
48. Ulmer-Franco AM, Fasenko GM, O'Dea Christopher EE. Hatching egg characteristics, chick quality, and broiler performance at 2 breeder flock ages and from 3 egg weights. *Poult Sci* 2010;89:2735–2742.
49. Tufft LS, Jensen LS. Effect of age of hen, egg weight, and sex on chick performance and lipid retention. *Poult Sci* 1991;70:2411–2418.
50. Lowenthal JW, Connick T, McWATERS PG, York JJ. Development of T cell immune responsiveness in the chicken. *Immunol Cell Biol* 1994;72:115–122.
51. Abdul-Careem MF, Hunter DB, Lambourne MD, Barta J, Sharif S. Ontogeny of cytokine gene expression in the chicken spleen. *Poult Sci* 2007;86:1351–1355.
52. Peters MA, Browning GF, Washington EA, Crabb BS, Kaiser P. Embryonic age influences the capacity for cytokine induction in chicken thymocytes. *Immunology* 2003;110:358–367.
53. Ariaans MP, van de Haar PM, Lowenthal JW, van Eden W, Hensen EJ et al. ELISPOT and intracellular cytokine staining: novel assays for quantifying T cell responses in the chicken. *Dev Comp Immunol* 2008;32:1398–1404.
54. Boyd AC, Ruiz-Hernandez R, Peroval MY, Carson C, Balkissoon D et al. Towards a universal vaccine for avian influenza: protective efficacy of modified vaccinia virus Ankara and adenovirus vaccines expressing conserved influenza antigens in chickens challenged with low pathogenic avian influenza virus. *Vaccine*. Elsevier Ltd; 2013;31:670–675.
55. Wu CC, Thiagarajan D, Lin TL. Research notes: ELISPOT assay for detection of antibody secreting cells to infectious bursal disease virus in chickens. *Poult Sci* 1998;77:662–665.
56. Williams MA, Bevan MJ. Effector and memory CTL differentiation. *Annu Rev Immunol* 2007;25:171–192.
57. Whitmire JK. Induction and function of virus-specific CD4⁺ T cell responses. *Virology*. Elsevier Inc.; 2011;411:216–228.
58. Remakus S, Sigal LJ. Memory CD8⁺ T cell protection. *Adv Exp Med Biol* 2013;785:77–86.
59. Beirão BCB, Fávoro C, Nakao LS, Caron LF, Zanata SM et al. Flow cytometric immune profiling of specific-pathogen-free chickens before and after infectious challenges. *Vet Immunol Immunopathol* 2012;145:32–41.
60. Bridle BW, Julian R, Shewen PE, Vaillancourt J-P, Kaushik AK. T lymphocyte subpopulations diverge in commercially raised chickens. *Can J Vet Res* 2006;70:183–190.
61. Chen H-Y, Cui P, Cui B-A, Li H-P, Jiao X-Q et al. Immune responses of chickens inoculated with a recombinant fowlpox vaccine coexpressing glycoprotein B of infectious laryngotracheitis virus and chicken IL-18. *FEMS Immunol Med Microbiol* 2011;63:289–295.
62. Gupta A, Ahmed KA, Ayalew LE, Popowich S, Kurukulasuriya S et al. Immunogenicity and protective efficacy of virus-like particles and recombinant fiber proteins in broiler-breeder vaccination against fowl adenovirus (FAdV)-8b. *Vaccine* 2017;35:2716–2722.
63. Taeibpour MJ, Dadras H, Nazifi S, Afsar M, Ansari-Lari M. Evaluation of blood monocyte and lymphocyte population in broiler chicken after vaccination and experimental challenge with Newcastle disease virus. *Vet Immunol Immunopathol* 2017;190:31–38.
64. Kongsuwan K, Johnson MA, Prideaux CT, Sheppard M. Identification of an infectious laryngotracheitis virus gene encoding an

- immunogenic protein with a predicted M(r) of 32 kilodaltons. *Virus Res* 1993;29:125–140.
65. Godlewska R, Kuczkowski M, Wszyńska A, Klim J, Derlatka K *et al.* Evaluation of a protective effect of in ovo delivered *Campylobacter jejuni* OMVs. *Appl Microbiol Biotechnol* 2016;100:8855–8864.
66. Kapczynski DR, Martin A, Haddad EE, King DJ. Protection from clinical disease against three highly virulent strains of Newcastle disease virus after in ovo application of an antibody-antigen complex vaccine in maternal antibody-positive chickens. *Avian Dis* 2012;56:555–560.

Five reasons to publish your next article with a Microbiology Society journal

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as 'excellent' or 'very good'.
5. Your article will be published on an interactive journal platform with advanced metrics.

Find out more and submit your article at microbiologyresearch.org.